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Gap Junctions between Electrotonically Coupled Cells in Tissue Culture and in Brown Fat

(BHK cells/electron microscopy/freeze replicas/mouse/morphology)

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ABSTRACT Although circumstantial evidence has suggested that gap junctions mediate intercellular electrotonic coupling, it has not been possible in most tissues to exclude the involvement of other, coexisting cell junctions. We have made an electron microscopic study of replicas of frozen-fractured BHK21 cells (from tissue culture) and of brown fat cells of newborn mice. Both of these cell types are known to exhibit intercellular electrical coupling. In each case, the only junctions found between the cells are small macular gap junctions (less than 1 μm in diameter) characterized by clusters of 6-nm (60 Å) particles or depressions on membrane cleavage faces. Several replicas confirm the association of these particles and depressions with regions of narrowing of the intercellular space, i.e., with the sites of cell junctions. We have also determined the frequency of occurrence of gap junctions on the membrane cleavage faces of both cell types. Gap junctions occupy about 1–2% of the surface area of brown fat cells, but only 0.05% of the surface area of BHK21 cells. These observations indicate that gap junctions, when they are the only intercellular junctions present, are sufficient to account for electronic coupling between cells.

Circumstantial evidence accumulated over the last few years suggests that gap junctions are the morphological specializations responsible for cell-to-cell electrotonic coupling (1–3). The results are equivocal, however, because the cells studied are also joined by other junctions: by *zonulae* or *maculae adherentes* (desmosomes), or by *zonulae occludentes* (tight junctions) in vertebrates (4), and by septate junctions (septate desmosomes) in invertebrates (5, 6). Perhaps the only case in which a gap junction is the sole identified intercellular contact between coupled cells is that of the septal synapse of the crayfish (3). Dreifuss *et al.* have shown that electrical coupling between heart cells can still be observed after the desmosome-like components of the intercalated discs have been disrupted by treatment with medium containing a low concentration of calcium ion (7). We have attempted to test directly the role of the gap junction in electrical coupling by studying tissues in which no junctions other than the gap junction have been found. In the present communication, we report the results of a study of cultured cells (BHK21 cells, derived from baby-hamster kidney fibroblasts) and of interscapular brown fat of newborn mice. Electrotonic coupling in these cells has been demonstrated by Furshpan and Potter (8) and by Sheridan (9, 10), respectively. In spite of careful electron microscopic investigations, neither tight junctions nor desmosomes have been described in either system. The use of freeze-cleaving (11, 12), however, has now allowed us to clearly demonstrate the presence of small, but characteristic, gap junctions in both types of cells.

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MATERIALS AND METHODS

The BHK21 cells were obtained through the kindness of Dr. E. Furshpan and had originally been derived from the American Type Culture Collection. The cells were grown to near confluence on glass coverslips in Dulbecco's medium. For conventional electron microscopy, they were fixed in osmium–glutaraldehyde (13), postfixed in OsO_4, stained *en bloc* in uranyl acetate (2, 4), and embedded in Epon–Araldite. For freeze-cleaving, coverslips bearing the cells were immersed in 2% glutaraldehyde in Earle's balanced salt solution, rinsed in saline, and stored at 4°C for at least 1 hr in 25% glycerol solution in Earle's medium. Samples were then removed from the coverslips by gentle scraping with a spatula. Large sheets of cells could be readily lifted off the glass surfaces and deposited with minimal disruption as "wads" on standard Balzers specimen holders. They were then frozen in liquid Freon 22 cooled to its freezing point in liquid nitrogen. Fracturing and replication were performed in a Balzers Freeze Etcher at −150°C (no etching). The platinum-shadowed carbon replicas were freed from the cells in Clorox, washed in distilled water, and mounted on celloidin-covered grids for examination in the Siemens IA electron microscope operating at 80 kV with 30-μm objective apertures.

The interscapular brown fat of 2-day-old mice was fixed either in Karnovsky's mixture (14) at full strength or diluted to 50% or 25% strength, or in 2% glutaraldehyde in Earle's saline. For conventional electron microscopy, the tissues were postfixed in OsO_4–collidine, treated with uranyl acetate, and embedded in Araldite (15). Thin sections were stained with lead citrate (16). For freeze-cleaving, the aldehyde-fixed tissues were placed in 25% glycerol in saline for at least 1 hr before etching in a Bullivant-type apparatus (17) (Wolken and Revel, unpublished) or in a Balzers Freeze Etcher, operated as described above. Before digestion in Clorox, the tissues were defatted overnight at room temperature in dimethyl formamide.

RESULTS

Electron microscopic study of thin sections shows that the overlapping portions of BHK21 cells are usually separated by an extracellular space some 500 Å wide. Occasionally, the space narrows abruptly and the plasma membranes of adjacent cells appear to come into contact or near contact (Fig. 1, insert). Because the area of such close apposition is often small (100–200 Å), it is usually not possible to determine

the nature of the contact specialization without analysis by serial sectioning or specimen tilting. Favorably oriented contacts appear to be small gap junctions, however, as the outer leaflets of the apposed membranes are separated by a space of 20–30 Å. Very infrequently, larger gap junctions of typical morphology are encountered. It has not been possible to demonstrate the presence of *zonulae occludentes* or of adherent junctions in any of the cultures.

The use of freeze-cleaving allows extensive views of cell-membrane fracture faces, which are more favorable for a study of junctions than are the membrane profiles visualized in sectioned material. Biological membranes apparently fracture along planes within their interiors, exposing two complementary fracture faces, one rough (A face) and the other smooth (B face) (18, 19). Replicas of junctional areas of BHK21 cells show agglomerations of closely packed particles on the exposed A faces of the membranes (Figs. 1 and 2) and depressions or pits on B faces (Fig. 3). Fortunate fractures, such as that shown in Fig. 4, clearly demonstrate that pits and particles are associated with regions where the normal intercellular space is narrowed, i.e., with regions of intercellular contacts. The arrays are quite small, usually

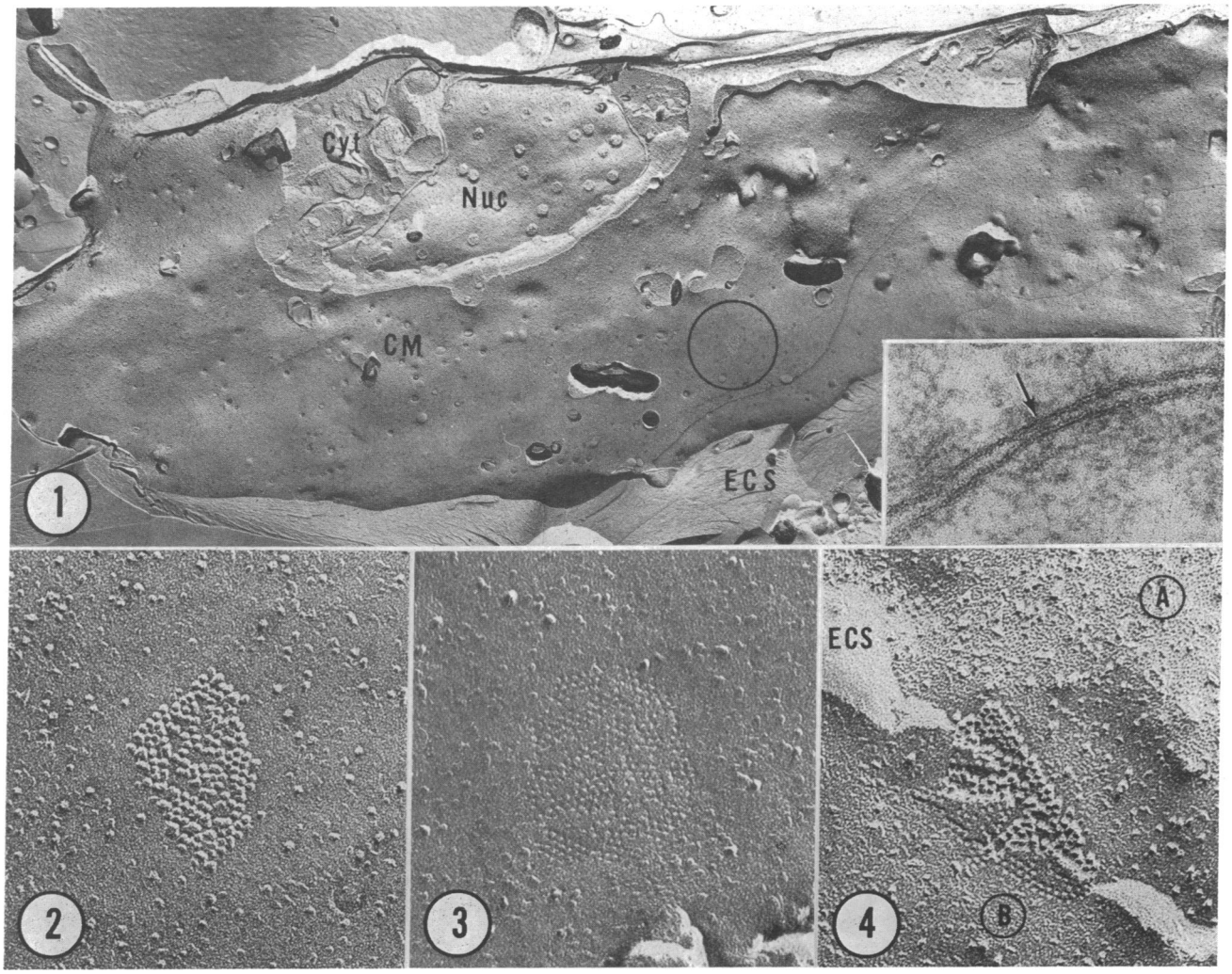

FIG. 1. Replica of a freeze-cleaved BHK21 cell. The fracture reveals a large area of cell membrane (*CM*) of a single cell. In the upper-left portion of the micrograph, a deeper fracture plane shows the nucleus (*Nuc*) with nuclear pores surrounded by cytoplasm (*Cyt*). Only one small gap junction (*circle*) is found on the extensive membrane-face exposed. No other membrane specializations representing other junctions, e.g., *zonulae occludentes* or desmosomes, are evident. *Insert.* Conventional electron micrograph of BHK21 cell membranes in a region of contact. The cell membranes of the two apposed cells approach each other and form a small focal junction (*arrow*). The exact nature (gap or tight?) of the junctional area cannot be clearly determined with this technique. *ECS* = extracellular space. ×18,000; *Insert* ×140,000.

FIG. 2. High-magnification micrograph of the area encircled in Fig. 1. The A face (particulate face) of the membrane includes a macular gap junction, characteristically composed of several groups of particles. Each group contains about 5–20 particles, and is separated from other groups by areas devoid of structures. The particles measure about 60 Å in diameter and have a center-to-center spacing of about 80 Å. The particle-poor halo around the junction is also apparent. ×150,000.

FIG. 3. High-magnification micrograph of the B face (pitted face) of a freeze-cleaved gap junction. Notice that the organization of pits into small domains is similar to that described for the particles on the A face (Fig. 2). BHK21 cell. ×150,000.

FIG. 4. High-magnification micrograph of a freeze-cleaved gap junction. The association of particles and pits with the junctional region is shown by the abrupt narrowing of the extracellular space. In conventional electron microscopy, this is represented as the 20–30 Å close approximation of the cell membranes typical of gap junctions. *A* = A face; *B* = B face; *ECS* = extracellular space. BHK21 cell. ×150,000.

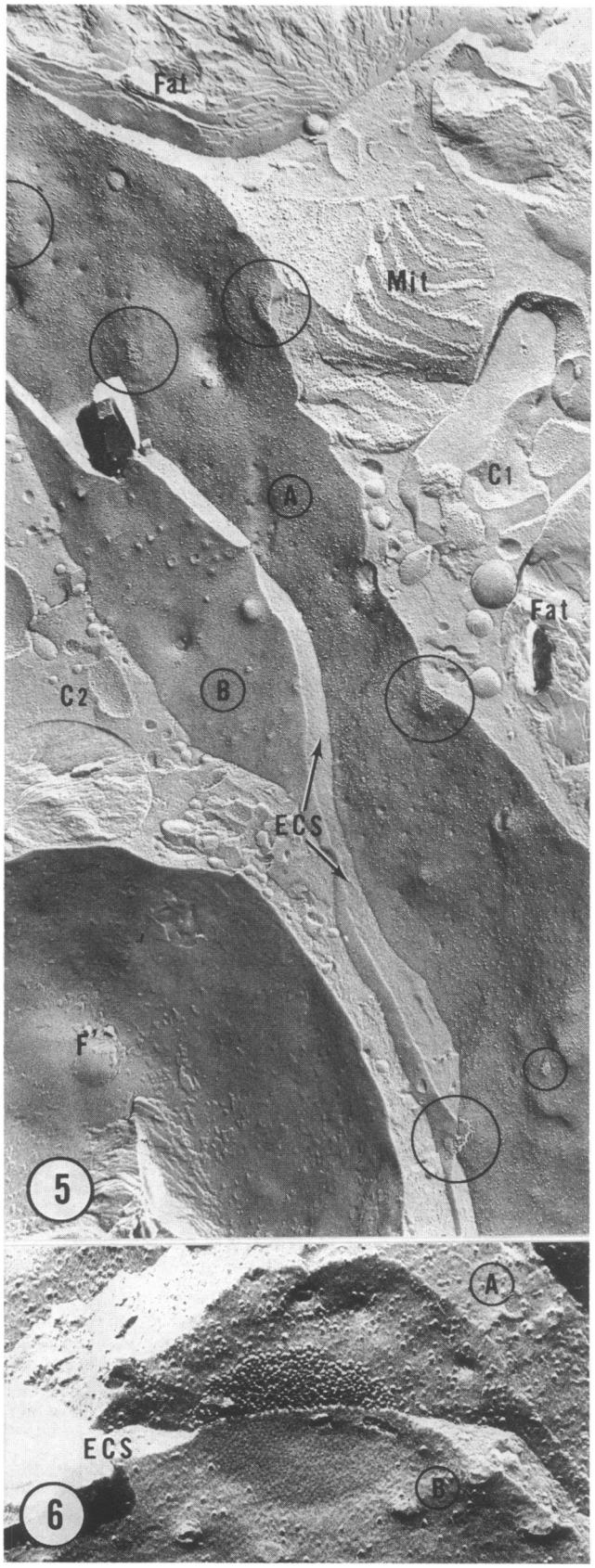

FIG. 5. Replica of freeze-cleaved interscapular brown fat from a 2-day-old mouse, showing contact between two cells (*C1* and *C2*) separated by a small amount of extracellular space (*ECS*). The exposed membrane face of cell 1 shows small, but numerous, gap junctions (*circles*). The relationship of particles and pits to the junctional region is evident in the lowest circle. Fracture planes through the fat inclusions (*Fat*) suggest a lamellar orientation of the lipid molecules. *F'* = fat vacuole from which contents have been fractured away; *Mit* = mitochondrion; *A* = A face; *B* = B face. ×32,000

FIG. 6. High-magnification micrograph of a freeze-cleaved gap junction between brown fat cells. Both particulate (*A*) and pitted (*B*) faces are exposed and, again, their association with the region of intercellular contact is indicated by the localized narrowing of the extracellular space (*ECS*). ×86,000.

0.1–0.2 μm in diameter; occasionally, however, arrays as large as 1 μm in diameter can be found. The contact specializations closely resemble other gap junctions studied by freeze-cleaving, with particles each about 60 Å wide separated by a center-to-center distance of about 80 Å (2, 20). The particle-poor halo described by McNutt (21) in heart muscle is also present. The only unusual, but constant, feature is the loose association of 5–20 particles or pits into domains, separated from differently oriented domains by small areas devoid of structure.

The amount of the cell surface involved in gap junctions has been determined by measuring the ratio of junctional to nonjunctional surface by stereological techniques (22). Only those cell surfaces containing junctions have been included in the measurements; these surfaces clearly represent regions of cell overlap. Other parts of the cell membrane may not be capable of forming intercellular junctions either because they are parts of free surfaces or because they are specialized for other functions. This method must accordingly yield too high an estimate for the junctional areas. Nevertheless, it is found that as little as 0.05% of the surface may be involved in gap junctions between BHK cells in culture. The small size of the junctions and the limited membrane area that they involve explain why it has been so difficult to establish their presence in sectioned material.

BHK cells, as an established cultured line, may have junctional properties that are very different from those of cells in the intact organism. It is, therefore, of interest to examine cells that have never been cultured. Studies on brown adipose tissue have shown that the fat cells are electronically coupled (9, 10) and no desmosomes or tight junctions have been found between the cells (9, 23). We have observed occasional gap junctions in sectioned material, but they are too rare and too small to allow complete and unequivocal characterization.

Freeze-cleaving of brown fat reveals numerous gap junctions, composed of small clusters of particles on the A faces, and of pits on the B faces of cell membranes (Figs. 5 and 6). The junctional maculae themselves are small, ranging in diameter from 0.3 to 1 μm, and are much more numerous than those found between BHK cells (Figs. 1 and 5). The characteristic particles of the junctional arrays are often, but not always, arranged in small domains separated by spaces devoid of structured elements. Since brown fat cells lack obvious polarity, all the plasma membranes can, *a priori*, be considered equivalent, and potentially capable of forming junctional contacts. It is, therefore, relatively safe to estimate the fraction of the surface of a cell involved in cell-to-cell interaction via gap junctions by an examination of randomly exposed membrane faces. We calculate that in brown fat, roughly 1–2% of the cell surface is devoted to gap junctions.

DISCUSSION

In both of the electronically coupled cell types examined here, the only intercellular contacts observed, whether by thin sectioning or by freeze-cleaving, are small gap junctions. Hyde and his collaborators (24) have examined the intercellular contacts of fibroblasts and cardiac myoblasts in primary cell culture, and have demonstrated focal close junctions among these coupled cells. It is possible that these junctions, and perhaps the focal junctions of electronically coupled embryonic cells (25, 26), are actually small gap junctions of the type described in this study.

There are many tissues where gap junctions are found between electrotonically coupled cells: the club endings on Mauthner cells of the goldfish brain (1), vertebrate hearts (2, 21), mammalian liver (20), smooth muscle (27), mucous cells of the stomach and absorptive cells of the small intestine (N. Spitzer†, personal communication), epididymis (unpublished), retinal pigment epithelium (unpublished), and lateral giant nerve fibers of the crayfish (3). Even in invertebrate epithelia, where septate junctions have been thought to be implicated in coupling (5), one can demonstrate the presence of gap junctions (6, 28, 29). This variety of cell types provides considerable circumstantial evidence that the gap junction mediates electrical coupling between cells. The present study strongly supports this hypothesis, and indicates moreover that coupling can occur when gap junctions are present alone. It must be recognized that it is very difficult to prove the absence of other types of junctions; nevertheless, our techniques should have demonstrated other junctions, if they did exist. In our replicas of brown fat, a built-in control is provided by the presence of capillaries whose endothelial cells are conjoined by clearly recognizable *zonulae occludentes*. The possibility remains, however, that there are other, as yet unknown, junctions responsible for intercellular coupling (30). With this qualification, we may therefore conclude that gap junctions are sufficient to account for electrical coupling between cells. This leaves open the questions of whether gap junctions are a requirement for electrotonic coupling, how they could mediate it, and, most important, what physiological purposes are served by coupling.

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